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Studies on the primary structure of short polysaccharides using SEC MALDI mass spectroscopy

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Abstract

The introduction of size-exclusion chromatography (SEC) analysis of polysaccharides prior to MALDI mass spectroscopy accounts for the determination of the molecular mass of the repeating unit when neutral homopolymers are investigated. In the case of natural polysaccharides characterised by more complicated structural features (presence of non-carbohydrate substituents, charged groups, etc.), this mass value usually is in agreement with more than one sugar composition. Therefore, it is not sufficient to give the correct monosaccharidic composition of the polysaccharide investigated. To solve this problem, MALDI spectra were recorded on the permethylated sample and post-source decay experiments were performed on precursor ions. In this way, the composition (in terms of Hex, HexNAc, etc.), size and sequence of the repeating unit were determined. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS) was first described in 1988 [1]. Since then it has been widely used to produce intact molecular ions from compounds with high molecular weight and low dispersity index, such as proteins, oligosaccharides, oligonucleotides and polymers obtained by anionic polymerisation. The application of this technique to the analysis of samples with a high dispersity index, such as polysaccharides and industrial polymers, required the introduction of the SEC MALDI technique [2–4] wherein the samples are fractionated by SEC (size-exclusion chromatography), in order to decrease their polydispersity to values compatible with MALDI analysis (i.e., < 1.1), prior to MS analysis. In this way it is possible to make a direct measurement of the molecular weight of the sample with the advantage of greater accuracy for non-standard samples. Moreover, the introduction of the delayed extraction technique [5] improved the resolution of MALDI spectra with the

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consequence that it is now possible to separate peaks differing by only one monosaccharide residue in a mass range up to 20–30,000 Da. However, up to now, applications of the SEC MALDI technique to polysaccharides have been limited to the determination of their molecular weight distribution.

In this paper we report the application of SEC MALDI MS and post-source decay (PSD) analysis for the structural determination of microbial polysaccharides. In general, the first step in the elucidation of the primary structure of a polysaccharide is the determination of the monosaccharidic composition of the repeating unit (in terms of Hex, HexNAc, etc). This is still today a tedious and troublesome task involving chemical degradation of the polysaccharide, gas or liquid chromatography and comparison with standard samples. Furthermore, once the monosaccharide composition is obtained, the next step in the structural elucidation is the determination of the sequence in the repeating unit. The purpose of this paper is to present the strategy developed in our laboratories for obtaining the molecular weight, the monosaccharide composition and the sequence of the repeating unit of bacterial polysaccharides by using SEC MALDI MS and PSD analysis. The study involved homopolysaccharides of relatively high molecular weight, which were investigated by SEC MALDI MS. The determination of the composition, size and sequence of the repeating unit of an anionic polysaccharide required the use of SEC MALDI MS together with PSD analysis.

The PSD method, an extension of MALDI MS, allows one to observe and identify structurally informative fragment ions from decay of a precursor ion that takes place in the field-free region after leaving the ion source. Highly sensitive structure analysis of various biological polymers has been demonstrated using this technique, the capabilities of which in sequence analysis of native and permethylated oligosaccharides were reported earlier in several studies [6–10].

2. Experimental

Materials.—Dextran standards from *Leuconocostoc mesenteroides* were purchased from Fluka Bio Chemika (Switzerland). Pullulan standards were obtained from Shodex and 2,5-dihydroxy benzoic acid (DHB) was from Sigma (St. Louis, MS, USA). The exopolysaccharide produced by *Pseudomonas flavescens* strain B62 (EPS B62) was isolated and purified as previously described [11].

MALDI mass spectrometry.—MALDI spectra were obtained by use of a Voyager DE mass spectrometer or on an STR instrument equipped with a reflectron system (both from Perseptive Biosystem, Framingham, MA). Both instruments were fitted with a pulsed nitrogen laser (337 nm) with a 3 ns pulse duration. External calibration was performed in all cases. In linear mode, 2,5-dihydroxybenzoic acid (DHB) (80 g/L in water–MeCN) was used as a matrix using the dried drop technique [12] with a total amount of sample of about 100 ng.

The PSD MALDI technique has been described in detail previously [13–18]. The method is based on mass analysis of product ions from unimolecular or collision-induced decay taking place in the first field-free drift path of a reflectron time-of-flight mass spectrometer [19]. In the PSD mode, spectra were measured at 20 kV acceleration voltage and the reflectron voltage was decreased in successive 5% steps. PSD calibration was performed using the precursor ion signals. Samples were prepared by premixing 1 μL of polysaccharide solution (100 pg) and 1 μL of DHB matrix solution (100 mg/ml dissolved in CH_3OH). The samples were allowed to dry at room temperature.

Collision-induced dissociation mass spectra were obtained using He as collision gas.

Methylation of the EPS B62.—Methylation of the sample was performed according to the modified Hakomori [20] method using potassium methylsulphinylmethanide [21].

3. Results and discussion

The MALDI mass spectra of two SEC fractions of dextran and pullulan are reported in Fig. 1(a) and (b), respectively. Peaks corresponding to sodiated molecular ions are present and, although the two polymers contain only glucose, the differences between

homologous peaks in the two spectra are 162 and 486 Da, respectively, which correspond to the molecular weight of the repeating unit of the two polysaccharides. In fact, the repeating unit of dextran is composed of a glucose unit, while that of the pullulan is a trisaccharide constituted of three glucose residues.

A much more complicated case is illustrated in Fig. 2, where the MALDI mass spectrum of the native extracellular polysaccharide produced by the bacterium *P. flavescens*, strain B62 [11] (EPS B62), is shown. Although this polymer is characterised by having a relatively low molecular weight, the spectrum

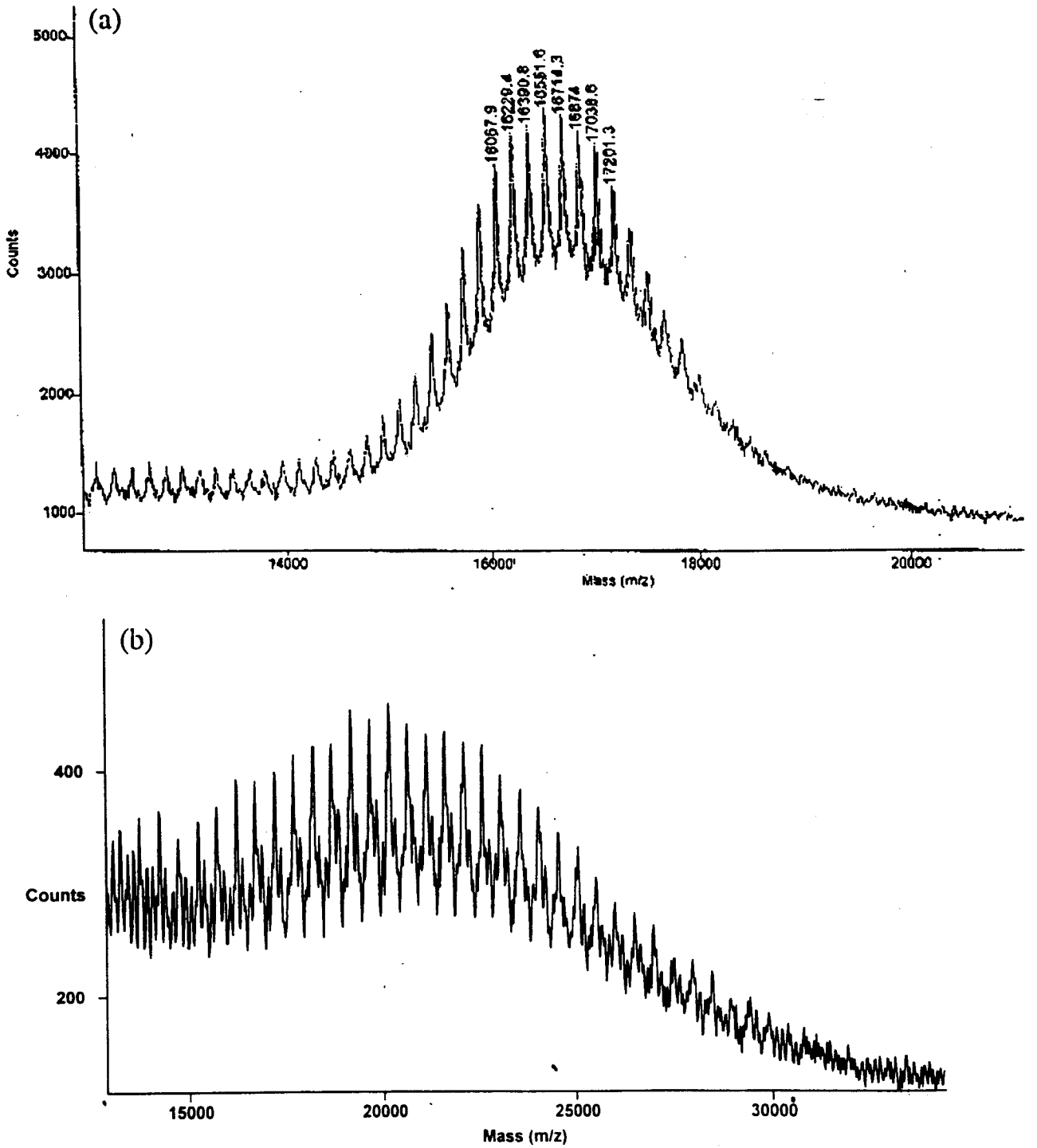


Fig. 1. (a) MALDI mass spectrum of a dextran SEC fraction. (b) MALDI mass spectrum of pullulan (20 kDa).

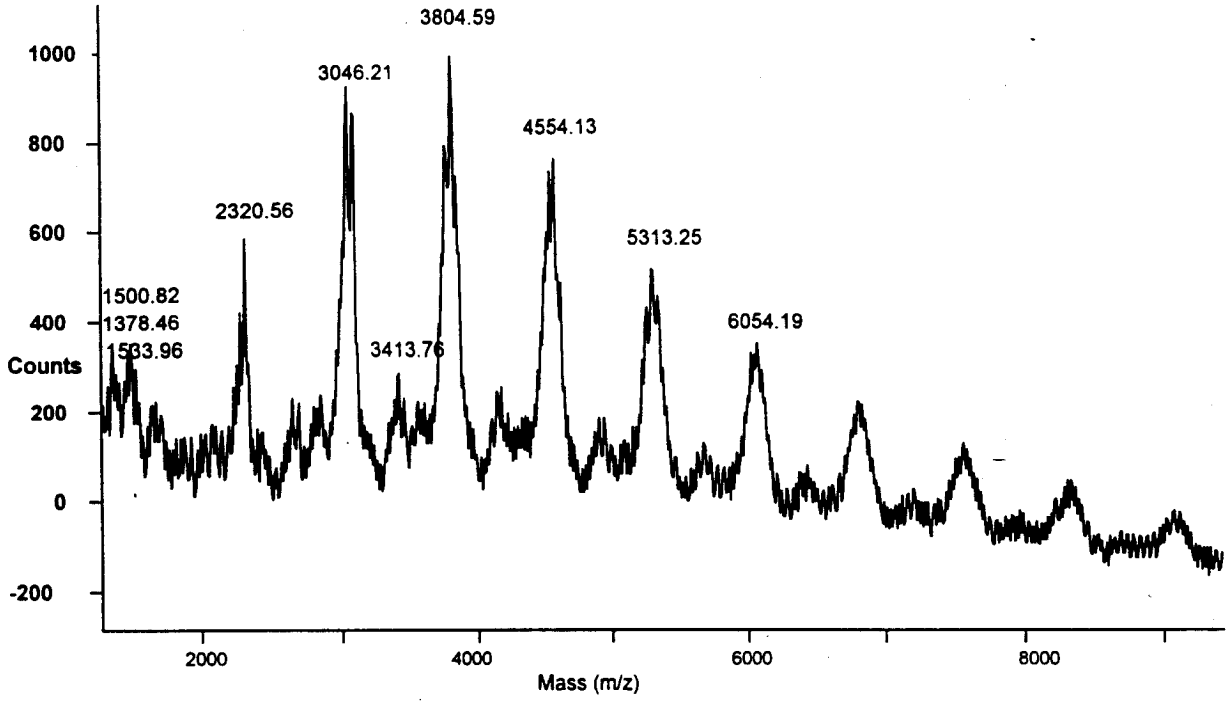


Fig. 2. MALDI mass spectrum of the native EPS B62.

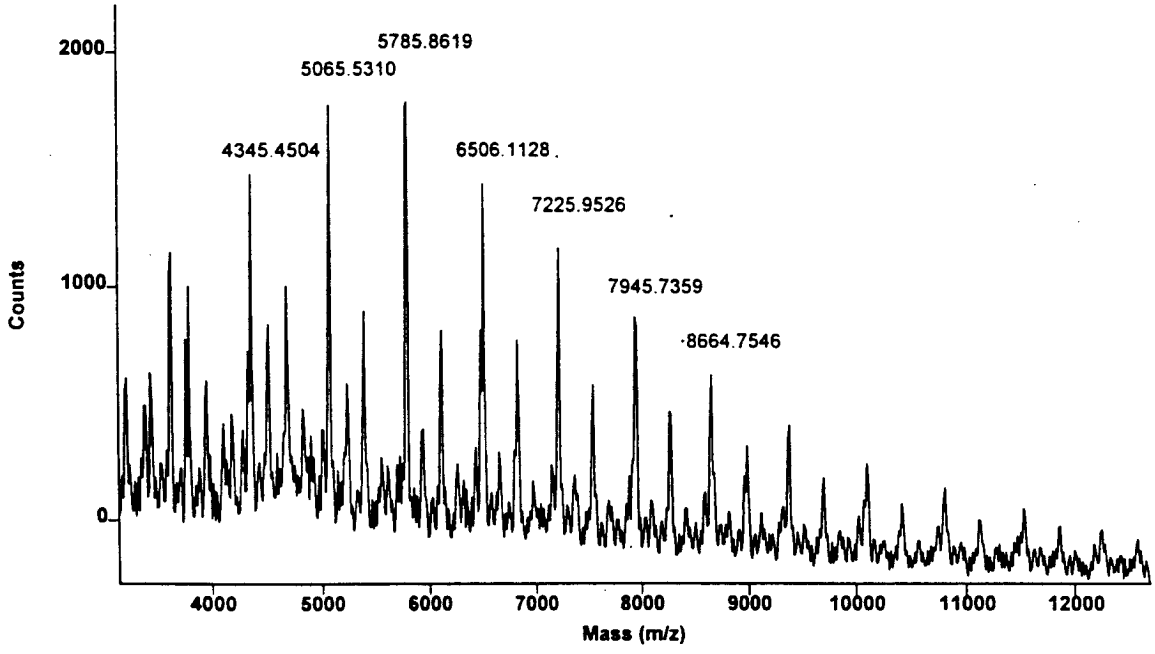


Fig. 3. MALDI mass spectrum of deacetylated EPS B62.

is characterised by a poor resolution, with heterogeneous peaks (see enlargement in Fig. 2), from which it is not possible to have any information about the molecular weight of the repeating unit. The peak heterogeneity is caused by two factors: the presence of carboxylate groups and of a random acetylation pattern. To overcome this problem, the EPS B62 was deacetylated and converted to its acidic form by using an ion-exchange resin. These modifications led to an accurate and sensitive MALDI analysis of EPS B62. In the

spectrum (Fig. 3), the peaks corresponding to molecular ions of different polysaccharide chains are separated by 718 Da, which is then the molecular mass of the repeating unit. In Fig. 3 the presence of other mass signals besides the ones expected can be explained by taking into account degradative processes due to the alkaline conditions of the deacetylation reaction. Several monosaccharidic compositions are in agreement with this repeating unit mass value, as shown in Table 1, where the output of the Carbo Mass program [22] is reported.

In order to determine the correct monosaccharidic composition, an aliquot of the polysaccharide was permethylated and then analysed by MALDI MS. The MALDI spectrum of the derivatised sample, reported in Fig. 4, shows a series of peaks with a mass difference of 872 Da, corresponding to the molecular weight of the permethylated repeating unit of the polysaccharide. Relating the MALDI mass spectrum of the native polysaccharide with the one recorded on the deacetylated sample, it is possible to establish that the acetylation degree is about 80%, by comparing

Table 1
Monosaccharide compositions matching molecular weights obtained by MALDI MS calculated using the Carbo Mass program [a]

Set A: molecular mass 718 Da								Set B: molecular mass 872 Da							
Hx	DH	Ha	Pt	C3	AG	GN	LU	Hx	DH	Ha	Pt	C3	AG	GN	LU
2	1	0	0	0	0	0	1	2	1	0	0	0	0	0	1
1	1	1	0	1	0	0	0	1	1	1	0	1	0	0	0
1	0	1	1	0	0	0	1	1	0	1	1	0	0	0	1
1	0	0	0	1	0	2	0	0	0	4	0	0	0	0	0
0	0	2	1	1	0	0	0	0	0	2	1	1	0	0	0
1	2	0	2	0	0	0	0	1	2	0	2	0	0	0	0
0	0	0	3	0	0	2	0	0	0	0	2	2	0	0	0
0	1	1	3	0	0	0	0	0	1	1	3	0	0	0	0

[a] Abbreviations: Hx, hexose; DH, deoxyhexose; Ha, hexuronic acid: Pt, pentose; C3, lactylmannose; AG, acetylglucosamine; LU, lactylhexuronic acid.

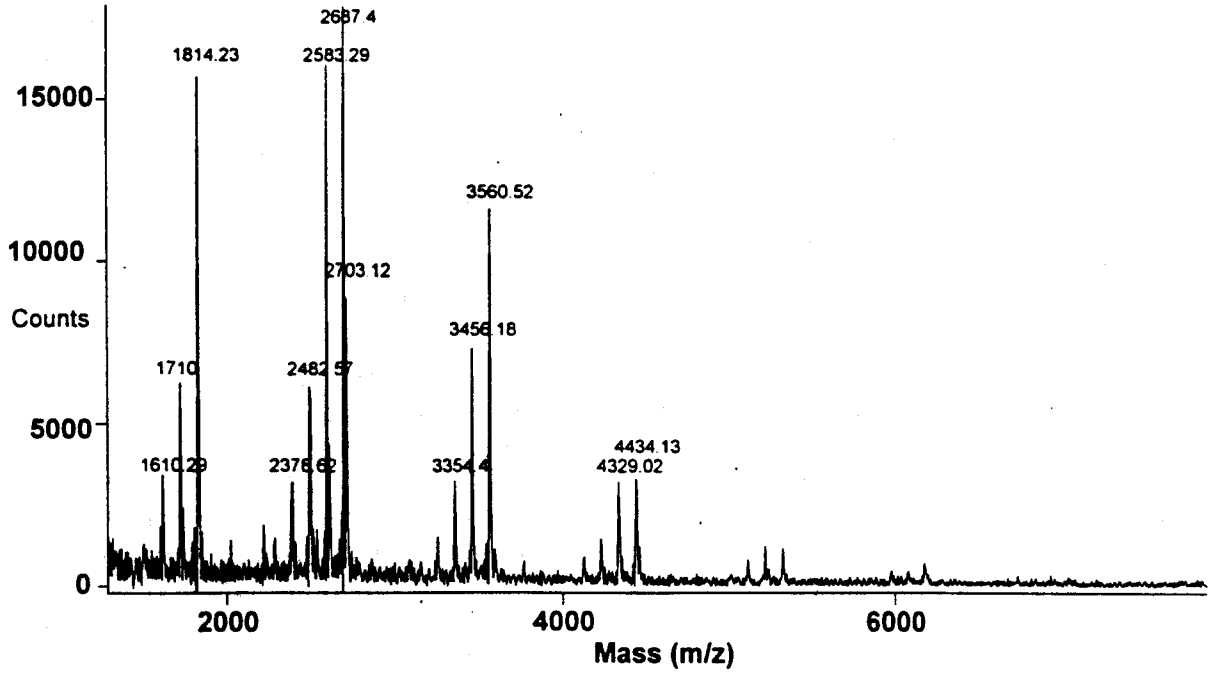


Fig. 4. MALDI mass spectrum of permethylated EPS B62.

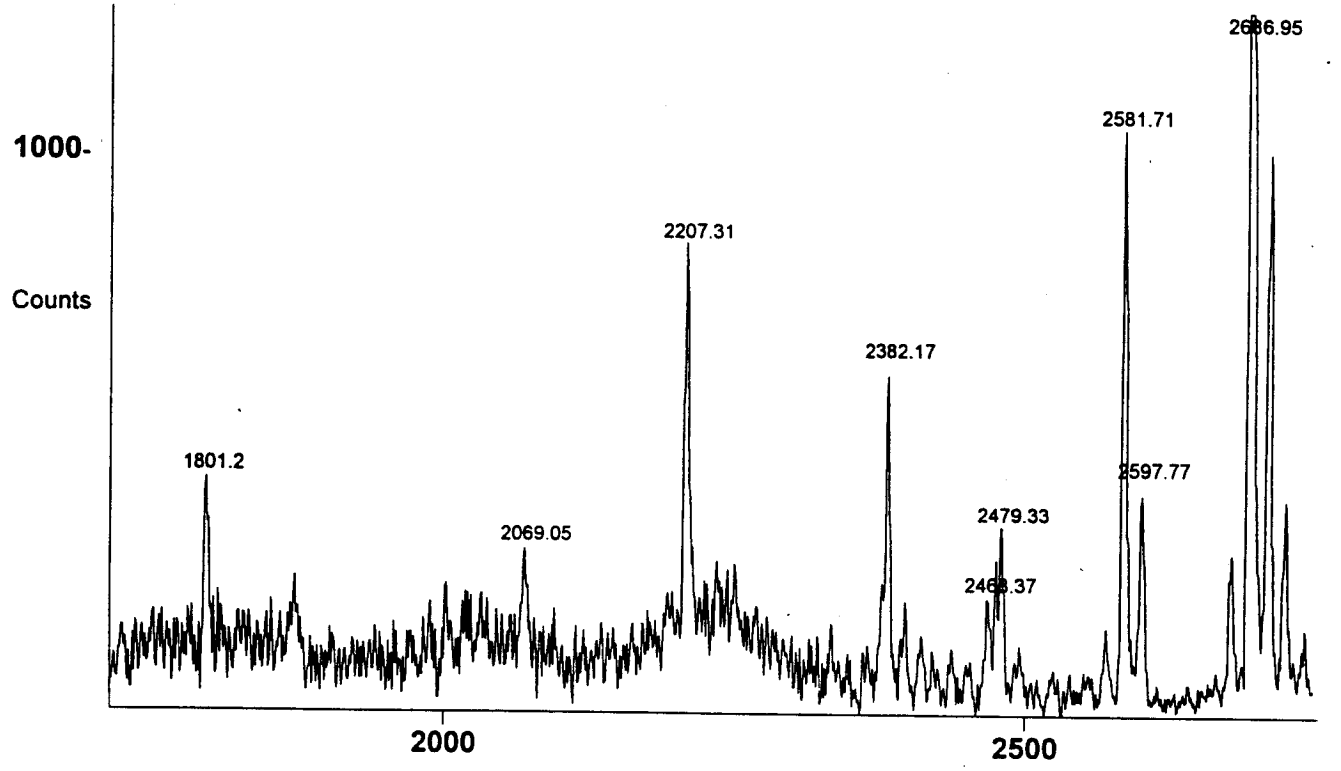
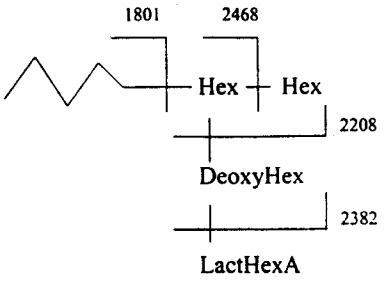


Fig. 5. PSD MALDI mass spectrum of permethylated EPS B62.

the masses of analogous peaks. The output of the Carbo Mass program is reported in Table 1. Also in this case, several different monosaccharidic compositions match this second mass value of 872 Da. Comparison of the two sets of compositions reported for the native and the methylated polysaccharide (Table 1) narrowed the feasible composition from eight to six different possibilities. Therefore, it was not possible to unambiguously establish the size of the repeating unit of the EPS B62 in terms of type of sugar residues (Hex, HexNAc, etc.). Losses of 104 Da from peaks corresponding to molecular ions at m/z 1814 + n872 can be explained by the well-known beta elimination reaction that occurs in the permethylation step [23]. The value of 104 Da can only account for a non-carbohydrate substituent linked to the C-4 of the uronic acid residue. Furthermore, multiple losses of the 104 Da fragment from the same parent peak can be explained only if the uronic acid is at a non-reducing end position, implying the existence of side chains with the substituted uronic acid as terminal residue. Among the most common substituents, the lactyl methyl ester accounts for a mass loss of 104 Da. After these considerations, only two sets of monosaccharidic compositions, compatible with both the native and permethylated polymer, contain a uronic acid substituted with a lactyl group. The correct sugar composition together with the sequence of the residues in the repeating unit were established by means of PSD analysis. The PSD MALDI mass spectrum of the trimer at m/z 2686 is reported in Fig. 5.

The PSD ions present a good signal to noise ratio and the relative signal intensities (i.e., intensity ratios between various peaks within each acquisition section) are reproducible both for different analyses of the same sample and for different sample preparations.

In addition to the $(M + Na)^+$ ion (m/z 2686), other intense peaks are present at m/z 2582, 2478, 2468, 2382, 2207 and 1801 Da. The first two peaks correspond to the losses of 104 and 208 Da, respectively, and are due to the beta elimination reaction [23] from the lactyl–hexuronic acid units. The other peaks in the spectrum are all produced by the cleavage of one glycosidic linkage. In fact, the two peaks at m/z 2468 and 2382 Da, which differ



Scheme 1. PSD fragmentation of permethylated EPS B62, the MALDI mass spectrum of which is shown in Fig. 5.

by 218 and 304 Da, respectively, from the precursor ion (2686 Da), denote that one hexose residue and the lactyl–hexuronic acid are terminal or reducing residues, while the peak at m/z 2207, being 174 Da apart from the signal at m/z 2382, indicates the presence of a deoxyhexose residue linked to the lactyl–hexuronic acid. This finding established that the correct composition of the repeating unit of the EPS B62 is Hex2–DeoxyHex–LactylHexA, as reported in the first line of Table 1. The next informative peak in the spectrum is at m/z 1801 Da corresponding to the mass of the dimer ion. All the reported data show that the sequence of the polysaccharide B62 is the one drawn in Scheme 1 and the results are in agreement with those already reported [11]. In addition, collision-induced dissociation (CID) mass spectra were measured (data not shown) and the resulting fragmentation pattern was exactly the same as that characterising the analysis obtained in absence of the collision gas.

Further PSD experiments were performed choosing the tetramer and the pentamer as precursor ions, at m/z 3558 and 4430, respectively. Both analyses gave results analogous to those obtained by studying the trimer ion (m/z 2686 Da).

4. Conclusions

MALDI mass spectrometry can be an excellent method in the structural analysis of polysaccharides when low-molecular-weight fractions are available.

In the case of homopolysaccharides, like dextran and pullulan, the MALDI mass spectrum of fractions obtained by SEC is sufficient for the determination of the composition and size of the repeating unit. When more structurally complicated polysaccharides have to be investigated, it is necessary to resort to a combination of techniques. For the polysaccharide reported in this study, the composition and sequence of the residues in the repeating unit were obtained by comparison of the MALDI spectra of the native and the permethylated samples, together with PSD experiments of precursor ions. This procedure complements procedures for characterising the molecular-weight distribution of large intact polysaccharides, such as SEC-MALLS [24] and analytical centrifugation [25].

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